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Temporal interactions of microbiota in longitudinal nasopharyngeal samples and association with lower respiratory tract infection

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DECLARATION

I, Brian Thabane Rambau (RMBBRI001), hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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DEDICATION

To Senior Prophet T.B Joshua, whose vision of Emmanuel TV has been a saving grace, and to my biological parents Julia and Elias for their encouragement throughout the hard times.

All glory to God who gave the grace for completion of this thesis.

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DISSERTATION ABSTRACT

During aetiology of respiratory illnesses, it is widely accepted that infection is preceded by nasopharyngeal (NP) colonisation with bacteria and that NP flora develop early in childhood (during the first year of life). The presence of multiple NP bacteria results in competitive and synergistic associations, however temporal organism interactions have rarely been explored due to limited availability of longitudinal data sets, and the complex statistical methods needed. This study aimed to identify, describe and quantify the temporal interactions existing between selected key bacteria colonizing the nasopharynx in young children (up to 1 year old), and to further compare these patterns in children who go on to develop pneumonia compared to those who do not.

The significance of the study, as well as the objectives of the study, methods and data analysis plan are outlined in the study protocol (Part A). A summary of what is currently known about NP bacterial species interactions is presented as part of the literature review (Part B). The primary aim of the literature review was to describe the prevalence of NP carriage of four NP colonizing bacteria of interest: S. *pneumoniae*, S. *aureus*, H. *influenzae* and M. *catarrhalis* in children, as well as identify any risk factors or confounding associations. The literature review furthermore aimed to identify previously described NP bacterial species interaction patterns, as well as providing a summary of statistical approaches previously employed in the studying bacterial interactions. A manuscript presenting the subsequent analysis of these data is included as Part C.

This study was a secondary data analysis of 760 infants enrolled in a birth cohort with NP swabs collected every two weeks for the first year of life and additionally at episodes of lower respiratory tract infections (LRTI). Kaplan-Meier estimates were used to visualize time to first carriage. Generalised estimating equations with a logit link and adjusted for repeated measures were used to estimate the time varying association of NP bacteria carriage with development of pneumonia, while enabling adjustment by key confounders. Markov multi state models (MSMs) were used to describe NP bacterial acquisition with age and estimation of clearance probabilities, new acquisition or persistent acquisition.

There were 760 individuals included in the analysis, with a total of 16,346 NP samples available and a median 364 person-days (IQR 346 – 365 person-days). S. *pneumoniae* was predominant, found in >55% of all samples and demonstrating carriage in >95% of individuals at least once by 12 months of age. S. *aureus* was both less common (25% of

samples and 88% of individuals) but also had a strikingly different pattern of first acquisition compared to the other three organisms, demonstrating a rapid increase in carriage prevalence until approximately four weeks and subsequently decreasing.

S. *pneumoniae* had the highest co-carriage prevalence overall with H. *influenzae* and M. *catarrhalis* (both 25%) but this varied by age category. In contrast, co-carriage with S. *aureus* was less prevalent with either S. *pneumoniae* (12%), H. *influenzae* (5%) or M. *catarrhalis* (6%). Co-carriage frequencies differed considerably by age category, at least partially reflecting the relative prevalence of carriage by age. Carriage and co-carriage rates were similar among those children that experienced LRTI compared to those that did not. Seasonal carriage varied, but to a small extent compared to variance by age.

Models adjusting for sex, site, season of birth and age found temporally sustained positive associations between the co-carriages of S. *pneumoniae* with H. *influenzae*, and M. *catarrhalis*, but no association with S. *aureus*.

Clear differences occur in the co-carriage patterns of S. *pneumoniae* with other organisms. The probability of acquisition of S. *pneumoniae* is modified by earlier carriage of H. *influenzae* or M. *catarrhalis*. Positive H. *influenzae* carriage increases the probability of acquisition of S. *pneumoniae* with transition probabilities from 0.15 (95% CI 0.14-0.17) to 0.36 (95% CI 0.17, 0.54) after 28 days of age, compared to the same period probability of acquisition of S. *pneumoniae* alone at 0.015 (95% CI 0.043-0.076) to 0.088 (95% CI 0.075-0.10). There is no difference in the clearance of S. *pneumoniae* related to H. *influenzae* carriage, but clearance of H. *influenzae* before 6 months of age is far less likely if coming from a state of co-carriage (probability between 0.04 - 0.07) compared to sole carriage (probability 0.23 - 0.12). The only evidence of differences in clearance probability in the models investigating S. *pneumoniae* and M. *catarrhalis* are in the probability of M. *catarrhalis* clearance before 28d which is 0.24 (95% CI 0.15 - 0.38) if carried alone and only 0.058 (55%CI 0.01 - 0.30) if carried with S. *pneumoniae*, though these confidence intervals overlap.

Through this modelling we found positive sustained interactions between S. *pneumoniae* and both H. *influenzae*, and M. *catarrhalis*, where models indicated that preceding carriage or colonisation with either H. *influenzae*, and M. *catarrhalis* may increase the risk of colonisation with S. *pneumonia*. Timing of carriage and overall prevalence of carriage are in line with other findings in similar populations with overall high exposure to S. *pneumoniae*,

H. *influenzae*, M. *catarrhalis* during the first year of life and rapid and early exposure to S. aureus. Carriage, co-carriage and transition frequency did not vary appreciably when comparing children who experienced LRTI in the first year of life compared to those who did not, suggesting that overall exposures are similar, but that further modelling is required to understand the specific timing of associations in relations to LRTI.

LIST OF ABBREVIATIONS

AOM	Acute otitis media
CRFs	Case report forms
DCHS	Drakenstein child health study
GEE	Generalised estimating equations
HI	Haemophilus *influenzae*
Hib	Haemophilus *influenzae* type-b
HIV	Human immunodeficiency virus
LMICs	Lower and middle income countries
LRT	Lower respiratory tract
LRTI	Lower respiratory tract infection
MC	Moraxella *catarrhalis*
MSMs	Multi state models
NP	Nasopharyngeal
OM	Otitis media
PCR	Polymerase chain reaction
PI	Principal Investigator
PRSP	Penicillin-resistant Streptococcus *pneumoniae*
PTB	Pulmonary Tuberculosis
qPCR	Quantitative polymerase chain reaction
RSV	Respiratory syncytial virus
SA	Staphylococcus aureus
SD	Standard deviation
SES	Socio-economic status
URI	Upper respiratory infection
URT	Upper respiratory tract
URTI	Upper respiratory tract infection
SP	Streptococcus *pneumoniae*
USA	United States of America
WHO	World Health Organization

WORD COUNT AND REFERENCES

Abstract: 897
Protocol: 2267
Protocol references: 571
Literature review: 4739
Literature review references: 1348
Manuscript: 4336
Manuscript references: 599
Whole thesis: 23 746

TABLE OF CONTENTS

TABLE OF FIGURES

Part B: Literature review

Part C: Manuscript

TABLE OF TABLES

Part B: Literature review

Part C: Manuscript

Part A: Protocol

Protocol Title: Identifying and quantifying organism interactions in longitudinal child health studies.

Introduction

In the aetiology of bacterial pneumonia and otitis media (OM), it is generally accepted that infection is preceded by nasopharyngeal (NP) colonization (Dunne et al. 2013). Several studies support that NP flora develop and establish early in childhood or in first year of life. Of the organisms that colonize the nasopharynx (i.e. those that develop and establish), the commonly studied are S. *pneumoniae*, H. *influenzae*, M. *catarrhalis* and S. *aureus*, all of which are associated with respiratory disease in children. However, colonization of the nasopharynx of young children is often asymptomatic (Murphy et al. 2009), and organism colonisation is known to co-occur. Colonisation timing or pattern can be impacted by risk factors, antimicrobial treatment and/or vaccination (Xu et al. 2012).

A body of literature exist on organism interactions, however temporal organism interactions have not been explored, especially regarding whether observed colonization patterns are resultant of the host's immunity or synergistic and antagonistic organism interactions. An understanding of the time dependent interactions in young children could provide insight into interpretation of estimated nasopharyngeal (NP) colonization thresholds and diagnosis of respiratory tract infections.

Research aims and objectives

The primary aim of this study is to identify, describe and quantify temporal interactions existing between selected pathogens occurring in the nasopharynx in young children (up to 1 year of life), and to compare these patterns in children who have an occurrence of lower respiratory tract infection (LRTI) in the first year of life compared to those that do not.

Specific objectives:

1. Describe the frequency, seasonality, and age related patterns of S *aureus*, S *pneumoniae*, H *influenzae* and M *catarrhalis* carriage from NP samples in infants less than 1 year.
2. Estimate the direction and magnitude of co-occurrence and temporal interactions between S *pneumoniae* and each of S *aureus*, H *influenzae* and M *catarrhalis*.
3. Compare the patterns of organism colonisation between infants who have experienced an episode of LRTI in the first year of life versus those who have not.

Background

Lower respiratory tract infections (LRTI) persist as the leading cause of morbidity and mortality in young children with an estimated incidence of about 0.22 episodes per child-year in low- and middle-income countries (LMICs) in 2010 (Rudan et al. 2013). Although LRTI does not follow a one-cause one-disease postulate, there are known more common disease causing organisms. Among pneumonia cases in a South African setting, respiratory syncytial virus (RSV) was the most common pathogen followed by influenza, at 29% and 17%, respectively. Moreover, the estimated incidence of community acquired pneumonia was 0.14 (705 554/5 041 132) for all acute lower respiratory infections (LRTI) and 0.4 (203 482/5 041 132) for RSV related pneumonia in the same setting (Rudan et al. 2013). Among young children globally, at least 50% are expected to be colonized with S *pneumoniae*, H *influenzae* or M *catarrhalis* at a given point in time and approximately 35% with S *aureus* (Pettigrew et al. 2008). All four organisms are known to cause disease, primarily respiratory tract infections, in early childhood, and so represent important species of study. Though organism carriage in the nasopharynx (NP) is not necessarily indicative of colonisation, nor of progression to disease, carriage is a necessary precursor (Anthony et al. 2012).

Studying carriage risk factors may offer an opportunity to intervene on respiratory disease by enabling one to alter or modulate exposure to predisposing factors, especially in pathogenesis of respiratory infections. Risk factors for NP colonisation by one organism may be similar or different to NP colonisation by other common NP colonisers. For instance, Pettigrew et al. (2008) showed that age, race and day care attendance were associated with colonisation by H *influenzae* but not with S *pneumoniae* or M *catarrhalis*. In addition to environmental and clinical risk factors, another factor that may alter progression to disease is co-occurrence or competing occurrence of organisms. Organism interactions have been studied for years including exploration of host, agent and environmental factors, i.e. whether or not these predispose one to nasopharyngeal (NP) colonisation and subsequent disease. Lewnard et al. (2016) was interested in whether observed organism interactions were a true reflection of interspecies interactions or merely just confounded results. Using longitudinal data of children aged between 2 – 30 months, the authors arrived at the conclusion that observed organism interactions are more likely to be due to interspecies interaction rather than simple confounding either by age differences or seasonal variations.

Previous work (Heinsbroek et al. 2015) has reported distributions in carriage of S *pneumoniae* among infants by maternal HIV exposure status (no difference), age (64.7% at 14 weeks of age compared to 27.1% at 52 weeks of age) and season (August peak), using longitudinal household data. Similarly, both S. *pneumoniae* and H. *influenzae* carriage have been report to peak during winter seasons (Lewnard et al. 2016). Further work by Lewnard et al. (2016) investigating possible confounding in analysis of organism interactions, found no confounding by age or season, but potential confounding by differences in socio-economic status (SES). In their data, investigators found that among a low SES population both S *pneumoniae* and H *influenzae* acquisition was earlier and with higher overall prevalence than compared to a high SES population at two months of age, noting that the groups came from different cultural backgrounds. Co-colonisation with S. *pneumoniae* and H. *influenzae* resulted in a 25% greater decline in S. *aureus* carriage in the children from the low SES households compared to the high SES households (Lewnard et al. 2016).

A number of studies investigating NP organism interactions have been done, but often describe contradictory findings (Murphy et al. 2009). Authors have described synergism between *S. pneumoniae* + H. *influenzae* and antagonism between both S *pneumoniae* + S *aureus* and H. *influenzae* + S *aureus* (Lewnard et al. 2016). Pettigrew et al (2008), however, describe an antagonistic association between H *influenzae* and S *pneumoniae*. Although there are slight age differences in the children between the studies, that alone does not appear to sufficiently explain the different findings. Further, the results suggest that additional organisms, for example, considering co-colonisation by M *catarrhalis*, substantially increased the complexity of evaluation. In Pettigrew et al (2008), it was found that when M *catarrhalis* co-colonised with H *influenzae* the antagonism between H *influenzae* + S *pneumoniae* reversed to synergism. Inverse associations were also observed between both S *pneumoniae* and H *influenzae* individually with S *aureus* (Pettigrew et al 2008). The degree of complexity in understanding even interactions between a few organisms calls for careful longitudinal studies and considered analysis.

Most of the literature estimating organism interactions are cross sectional study designs, with only a handful being longitudinal or repeated measures designs (Jacoby et al. 2007; Pettigrew et al. 2008; Rupa et al. 2012). While longitudinal designs have obvious strengths in assessing

temporal organism interactions, accounting for the variance from repeated individual participant samplings (i.e. serial observations or correlation) is important. Previously applied methods have varied, but primarily have been focused on generalized estimating equation (GEE), a 'population-averaged' model. For example, Pettigrew et al., (2008) followed infants 6 – 36 months old for a period of 1 year and used a GEE framework under a repeated measures logistic regression with an autoregressive correlation structure to estimate population odds ratios. Similarly, Rupa et al. (2012) modelled longitudinal data accounting for serial observations through an exchangeable correlation structure, again using a GEE framework. Unlike the GEE marginal models, Jacoby et al. (2007) applied a conditional multivariable mixed effects models to estimate pairwise interactions. Apart from GEE, exploratory analysis computing carriage frequencies are part of the early literature on NP colonisation and organism interactions. With cross-sectional data, interactions are typically estimated by unadjusted odds ratios from chi-squared test or Fisher's exact test. The timing of carriage events has also being explored by survival analysis methods.

Methodology

Study Design

This data for this secondary analysis is taken from the Drakenstein Child Health Study (DCHS) (Zar et al. 2014). DCHS is a population-based birth cohort study investigating early-life determinants of child health. Mother-infant pairs were enrolled at the time of infant birth and followed longitudinally for five years. The DCHS cohort includes >1000 mother-infant pairs, and follow-up is ongoing. The enrolled participants attended routine study visits at scheduled time points as well as unscheduled visits during sick episodes. A subset of the cohort elected to be enrolled into an intensively sampled group, who underwent 2-weekly visits during the first year of life. The samples for this analysis are from the intensively sampled sub-cohort.

Characteristics of the study population

The DCHS parent study recruited pregnant mothers in their second trimester a from peri-urban area in Cape Town, characterized by low socio-economic status (SES), informal housing, crowding and a high unemployment rate (Zar et al. 2015). Neonates and their mothers are followed for at least 5 years with some enrolled into an intensively sampled early

life cohort. Children received PCV13 and Hib immunizations from the primary health care, which are part of the South African routine immunization programme with the majority (>99%) of children receiving the full course of vaccination. HIV prevalence among mothers is 25% with only 2 HIV infected children (Zar et al. 2013).

Recruitment and enrolment

Research procedures and data collection methods

This study investigates selected measures from the subset of >800 infants participating in an intensively measured cohort during the first year of life. Study visits were conducted by trained study staff and data entered into REDCap database using standardised case report forms (CRFs). Primary outcome measures are from nasopharyngeal (NP) swabs that were taken at both routine study visits (every 2 weeks) and at episodes of lower tract respiratory infection (LRTI) identified by active surveillance. Collected NP swabs (Copan flocked swab, FLOQSwabsTM, COPAN Diagnostics, Murrieta, CA, United States) were immediately placed into 1ml skim milk-tryptone-glucose-glycerol (STGG), transported at 4 °C to the laboratory within 2hours of collection and frozen at −80 °C for later batch culture. Swabs were inoculated onto Mannitol Salt Agar (MSA) (National Health Laboratory Services, Green Point Media Laboratory Cape Town, South Africa) and incubated at 35◦C for 18–24h in room temperature air prior to measurement. The presence or absence of the S *aureus*, S *pneumoniae*, H *influenzae* and M *catarrhalis* in culture results, as well as the date of the sample, the infant date of birth, and a few key clinical or demographic parameters including sex, site, and date of LRTI are included in the set of analysis measures.

Data analysis

The opportunity to work on a very densely sampled cohort provides different possible study designs. In the first instance infants will be categorised in two groups, those who developed pneumonia in the first year and those that did not, and the prevalence and pattern of colonisation of the four identified organisms will be described. Kaplan-Meier and time to event methods will be used to describe time to first organism acquisition.

To describe the pairwise temporal interactions Chi-squared and Fisher's exact tests will be used to estimate the odds ratio (OR) of positive-positive interaction between pairs of visits, versus no association or positive-negative interaction. This preliminary exploration will need to be adjusted for infant age in order to account for possible confounding.

Generalised estimating equations (GEE) or binomial models for repeated measures will be used to estimate the time varying association of organism colonisation with the development of pneumonia, while enabling adjustment by key confounders.

Multi-state models will be developed to estimate the probability of state transitions where states are defined by the presence or absence of specified organisms in a given sample.

Research feasibility

The DCHS parent study has been approved by all appropriate ethics boards and permission to use a sample from the data has been granted. My proposed supervisor has experience with the proposed methods and is a regular collaborator with the DCHS PIs (Prof H Zar and Prof M Nicol).

Description of risks and benefits

As this is a secondary analysis of data that has already been collected and de-identified, there are no direct risks or direct benefits to participants in this study. There is a small risk of loss of confidentiality due to the presence of personal data. There are anticipated general benefits as the work may help inform the pattern and role of key organisms in early life in association to lower respiratory tract infection.

Informed consent process

Written informed consent in the parent study was obtained from mothers and renewed annually. Additional consent was obtained from fathers, as identified by mothers, when possible. The DCHS received ethical approval from Human Research Ethics Committee (HREC), Faculty of Health Sciences University of Cape Town, Stellenbosch University and the Western Cape Provincial Research Committee.

Privacy and confidentiality

The privacy of participants is ensured in the analysis as the sample dataset has been de-identified by the DCHS data management team. No personal participant identifiers such as ID numbers or names are available to researchers. Study data will further be maintained on a password protected computer.

Dissemination of research findings

The findings will submitted as a mini-dissertation in partial fulfilment of the Masters of Public Health degree from the University of Cape Town and may also be submitted for publication in a peer reviewed journal.

Public health significance

In addition to the benefits alluded to in the problem identification section above, an understanding of underlying temporal organism interactions in the nasopharynx may help predict how novel vaccines against these pathogens may influence the ecology in this niche. It may also provide an avenue for design of more effective strategies to combating respiratory infections (Dunne et al. 2013).

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Part B: Literature Review

Literature review objectives

The objectives of this literature review are to:

1. Describe the prevalence of nasopharyngeal carriage of four bacterial species (*Streptococcus pneumoniae, Staphylococcus aureus, Haemophilus influenzae* and *Moraxella catarrhalis)* in children in low- and middle-income countries, and explain any identified risk factors or confounding associations.
2. Identify and describe any organism interactions between the bacterial species.
3. Summarise statistical methods that have been applied previously to estimate either magnitude of, or direction of, organism interactions.

Search strategy

A search for relevant literature on organism interactions and nasopharyngeal carriage was conducted in PubMed in September 2018 using syntax constructed around the following search terms: upper respiratory tract; lower respiratory tract; Streptococcus pneumoniae; Haemophilus influenzae; Moraxella catarrhalis; Staphylococcus aureus; colonisation; children; interactions; pneumonia; nasopharyngeal colonisation.

Summary of the literature

The search resulted in 34 full papers which were reviewed. The search methodology is summarised in Supplement Figure 1 (Appendix) and further details are available in Supplement Table 1 (Appendix)

Bacterial nasopharyngeal carriage and risk factors

Variable nasopharyngeal (NP) carriage rates have been reported across different studies of varying populations, with the child's age appearing to modify carriage most strongly [1-13]. In a US cohort of children aged 6 - 36 months old [1], NP carriage prevalence per organism was 46% for *S. pneumoniae*, 7% for *S. aureus*, 32% for *H. influenzae* and 63% for *M. catarrhalis*. The authors of this study indicated that *M. catarrhalis* and *S. aureus* carriage was considerably below expected prevalence. Among Kenyan children between the ages of 12 – 59 months old, 50% of children who carried *S. pneumoniae* also co-carried *H. influenzae* [4]. Another Kenyan study which enrolled children between the ages of 3 - 59 months demonstrated the carriage rate for *S. pneumoniae* to be 66% [5]. A Gambian cohort of

children younger than 12 months of age (where NP samples were taken bi-weekly for the first six months of life and bi-monthly for remainder of the year) estimated that overall carriage rates of *S. pneumoniae, S. aureus, H. influenzae* and *M. catarrhalis* were 78%, 20%, 71% and 70%, respectively [6]. A similar study in Gambia [7] which enrolled children aged two months living in a peri-urban area, the carriage rates of *S. pneumoniae, S. aureus, H. influenzae* and *M. catarrhalis* were 62%, 50%, 30% and 32%, respectively. In Cape Town, South Africa among children who were characterised by poor socio-economic status, the overall carriage rate for *H. influenzae* was 46%, which was similar to the carriage rate estimated in children less than 12 months old at 44% [8]. A more recent study based in Cape Town on children with a median age of 36 months old and with suspected pulmonary Tuberculosis, the carriage rate of *S. pneumoniae, S. aureus* and *M. catarrhalis* were 42%, 22% and 64%, respectively [9]. Outside of sub-Saharan Africa, a Brazilian cohort of children 5 years or younger, of whom 53% had community acquired pneumonia the carriage rate of *S. pneumoniae* was 55% [10]. These data are summarised in Table 1.

Table 1. Bacterial species carriage rates in certain age groups and certain geographical regions

Bacteria prevalence	Study's geographic area and participants' age characteristics							
	USA[1] 6–12 months old	USA[2] 6-24 months old	Kenya[1] 12-59 months old	Kenya[2] 3-59 months old	Gambia[1] <12 months old	Gambia[2] 2 months old	South Africa[1] <12 months old	South Africa[2] median age 36 months
SP (%)	46%	30%	-	66%	78%	62%	-	42%
SA (%)	7%	-	-	-	20%	50%	-	22%
HI (%)	32%	12%	-	-	71%	30%	46%	-
MC (%)	63%	36%	-	-	70%	32%	-	64%
SP and HI co-carriage	-	-	50%	-	-	-	-	-

[1] and [2] indicates 2 different studies in the same country. SP – *S_pneumoniae;* SA - *S. aureus;* HI - *H. influenza*; MC - *M. catarrhalis*

A number of risk factors have been identified in children that are typically thought to be associated with organism carriage. These risk factors may include a social context, for example, day care attendance or the number of young children in a household. Clinical indicators may include a previous or current illness, as well as demographics, sex and age. In children aged 1 - 6 years old who had prior experience of penicillin-resistant *S. pneumoniae*

(PRSP) infection and who attended daycare, gender was not associated with any bacterial colonisation by either *S. pneumoniae, S. aureus, H. influenzae* or *M. catarrhalis*. Age, however was associated with colonisation by *M. catarrhalis* while cumulative time spent at the daycare centre was associated with colonisation by *H. influenzae* [11]. A similar lack of association between *S. pneumoniae* colonisation and gender was shown in children less than 5 years, in a Brazilian study [10]. This study showed that overcrowded housing was an important risk factor for *S. pneumoniae* colonisation especially in those individuals with a prior community acquired pneumonia. However, crowding, method of cooking and tobacco exposure were not associated with *S. pneumoniae, S. aureus, H. influenzae* or *M. catarrhalis* carriage in Venezuela, but were instead associated with poor nutrition [12]. In South African children hospitalised with Tuberculosis, those from a poor socio-economic background experienced higher rates of colonisation by *H. influenzae* compared to children from higher socio-economic backgrounds [11]. Among HIV-exposed neonates in Tanzania who had not received any pneumococcal conjugate vaccine (PCV), *S. aureus* colonisation was more prevalent in HIV-infected children compared to HIV-uninfected children. The converse applied for *S. pneumoniae* colonisation [15]. Moreover, the same study indicated that at 6 weeks of life colonisation by *S. aureus* was associated with urban area and having siblings younger than 10 years old. In contrast, at 6 months old only HIV infection remained associated with *S. aureus* colonisation [13]. This suggests that risk factors for *S. aureus* colonisation may be age dependent. Furthermore, at 3 months of age colonisation by *S. pneumoniae* and *H. influenzae* was associated with having siblings younger than 10 years old and colonisation by *M. catarrhalis* was associated with breast-feeding [13]. In preschool children aged 2 – 5 years old who had received antibiotics before undergoing an adenoidectomy for recurrent upper respiratory tract infections (URTIs), colonisation by at least one bacterial species (of *S. pneumoniae, S. aureus, H. influenzae* and *M. catarrhalis*) was associated with female gender and area of residence. Risk factors associated with individual bacterial colonisation included rural residence which was associated with a reduction in prevalence of *S. pneumoniae*; passive smoking and daycare attendance were associated with an increase in *H. influenzae*; female gender associated with an increase in *M. catarrhalis*; and residing in a rural area were associated with an increase in *S. aureus* [14].

Some studies have explored carriage rates specifically by disease status. Among children in South Africa younger than 5 years old, with Acute Otitis Media (AOM) the carriage rates of *S. pneumoniae, S. aureus, H. influenzae* and *M. catarrhalis* were 20%, 16%, 31% and 5%,

respectively [15]. These results were from cultured middle ear fluid samples rather than NP samples. In a much older cohort of children, median age 8.4 years old, who had a current lower respiratory tract infection (LRTI), NP carriage rates of *S. pneumoniae, S. aureus, H. influenzae* and *M. catarrhalis* were respectively 17%, 18%, 32% and 27% [16]. In a study of children 6 - 36 months old where NP samples were mostly collected within 7 days of an upper respiratory infection the overall carriage rates of *S. pneumoniae, S. aureus, H. influenzae* and *M. catarrhalis* were 46%, 7%, 32% and 63%, respectively [1]. The majority (96%) of these children had received at least one dose of PCV7 (vaccination available at time of research) at the time of study enrolment with as many as 95% not taking any antimicrobial drugs.

In Tanzania, HIV exposed neonates (six weeks old) of whom none had received any PCV, overall carriage rates of *S. pneumoniae, S. aureus, H. influenzae* and *M. catarrhalis* as 56%, 66%, 14% and 50%, respectively [13]. In healthy six month old children from USA [1], followed up until they were 24 months old, who had all received a dose of PCV-7 and other age appropriate vaccinations but no antimicrobial therapy, the carriage rates of *S. pneumoniae, H. influenzae* and *M. catarrhalis* were 30%, 12% and 36% respectively (see Table 1). Children with AOM had carriage rates of 53%, 48% and 43% respectively [17]. Moreover, the overall carriage rate by at least one of these bacterial species at healthy vs. at AOM visits were 57% vs. 90% [17] which suggests that the rate of NP bacterial colonisation can be expected to be higher in ill infants than in healthy children. These data are summarised in Table 2.

Table 2. Contrast of bacterial species carriage by infection and/or disease exposure

Bacteria specific prevalence	Illness and participants' age characteristics				
	AOM, <5years old, Middle ear fluid samples	LRTI, median age 8.4 years, NP samples	URTI 6-36 months old NP samples	HIV-exposed 6 weeks old, NP samples	AOM, 6-24 months old all received PCV7, NP samples
S. pneumoniae	20%	17%	46%	56%	53%
S. aureus	16%	18%	7%	66%	-
H. influenzae	31%	32%	32%	14%	48%
M. catarrhalis	5%	27%	63%	50%	43%

AOM: acute otitis media; LRTI: lower respiratory tract infection; URTI: upper respiratory tract infection, NP: nasopharyngeal

Healthy children may have a different set of risk factors compared to children with either a current or previous respiratory infection. In healthy Kenyan children of ages 3 – 59 months old who did not receive any pneumococcal vaccination, colonisation by *S. pneumoniae* was negatively associated with previous antibiotic use [5]. Meanwhile in Indonesian children aged 12 – 24 months old, the child's region of residence was associated with colonisation by *S. pneumoniae* and *M. catarrhalis* [17]. Furthermore, Israeli children <40 months old who did not receive any pneumococcal vaccination demonstrated that colonisation by *S. pneumoniae* was associated with their daycare attendance, having younger siblings and that they were older than 3 months; these three risk factors were all negatively associated with being colonised by *S. aureus* [18]. Although, maternal colonisation by *S. aureus* was associated with the child's subsequent colonisation by *S. aureus*, this was however not true for maternal carriage of S. *pneumoniae* and its subsequent colonisation in the child [18]. *Staphylococcus aureus* colonisation in children <6 months was not associated with either breastfeeding or the number of siblings [19].

Conjugate vaccine association with bacterial carriage before and after vaccination

A burden of disease related to vaccine-type *S. pneumoniae* persists in low- and middle-income countries (LMICs) despite certain serotypes of *S. pneumoniae* being effectively prevented by pneumococcal conjugate vaccine (PCV) PCV7, 9 and 13 [20]. The widespread introduction of PCVs raised some concerns over the possibility of a subsequent increase in *S. aureus* carriage and related disease post PCV vaccination [21]. This was due to the discovery of a negative association between *S. aureus* and *S. pneumoniae* [21]. Serotype replacement has been highlighted as one of the threats to conjugate vaccines, i.e. a biological phenomenon where NP colonisation by non-vaccine serotypes occurs post PCV vaccination. This risk might be heightened in neonates given the general consensus that NP flora develop in the first year of life and *S. aureus* carriage is typically highest among neonates. Risk generally declines with age. In South Africa, all children are expected to receive PCV and Hib vaccinations as part of the national immunisation programme. Hib is almost two decades old in the South African routine immunisation programme with PCV13 almost a decade old [22].

A number of studies have investigated the effects of the conjugate vaccines on NP carriage. In children aged 2 – 30 months, introduction to PCV7 did not affect *H. influenzae* or *S.*

aureus carriage rates [21]. A study compared children aged 12 – 59 months who received 2 doses of pneumococcal non-typeable *H. influenzae* protein-D conjugate vaccine (PHiD-CV) to children of the same age who received 2 doses of Hepatitis A vaccine, and showed that there was no significant increase in the carriage of NP bacteria such as *S. aureus* or *M. catarrhalis* due either to serotype replacement or the negative association between bacterial species [4]. Moreover, there was no increase in the carriage of *S. pneumoniae* or *H. influenzae* in either treatment group [4]. However, a different conclusion was reached by Van Gils et al. (2011) who showed that children who received a 2+1 dose of PCV (at the ages of 2, 4 and 11 months) there was evidence of temporary increase in *S. aureus* carriage at the age of 12 months while in the control treatment group (those who did not receive any PCV) there was a monotonic decrease in *S. aureus* carriage [23].

In Israel, children <40 months of age who had not been vaccinated with PCV, a negative association between vaccine-type strains of *S. pneumoniae* and colonisation by *S. aureus* was observed, additionally *S. aureus* carriage was higher in *S. pneumoniae* non-carriers compared to carriers and *S. pneumoniae* carriage was higher among *S. aureus* non-carriers compared to carriers [18]. Although a temporary increase in *S. aureus* was observed after adjusting for age [24], the authors argue that such an increase was not sufficient to support the hypothesis of an increase in *S. aureus* carriage post PCV vaccination due to the negative association between *S. aureus* and vaccine-type *S. pneumoniae*.

In South African children with AOM (3 months - 5 years) there was no significant difference in middle ear fluid carriage rates of *S. pneumoniae* and *H. influenzae* in those children who received a PCV7 dosage and those who did not [15]. Also, the number of PCV7 doses received by the child were not associated with nasal carriage of *S. pneumoniae*, vaccine-type and nonvaccine-type, or *S. aureus* in children aged between 6 weeks and 4 years [24]. Furthermore, even after adjusting for age, there was a temporary increase in *S. aureus* although this was found to be not sufficient to support the hypothesis that *S. aureus* carriage would increase after vaccination with PCV7 [24]..

Madhi et al. (2007) investigated the long term effects of PCV on NP colonisation by *S. pneumoniae* among both HIV-infected and uninfected South African children, and the interactions between pneumococcus species with other respiratory pathogens. Findings suggested that PCV9 and PCV13 increased NP colonisation by non-vaccine serotypes and reduced NP colonisation by vaccine serotypes [25]. Among South African children (mean

age 5.3 years) PCV was ineffective in reducing NP colonisation after 3 primary doses [22]. High *S. pneumoniae* and *H. influenzae* colonisation among HIV-infected South African children suggested that the burden of pneumococcal disease might be high among HIV infected children during periods of increased NP colonisation [25].

In a PCV7 dosing trial among 2 -30 months old children, the observed NP carriage rate of *S. pneumoniae* was 70% [21], while in children aged 6 weeks - 4 years old who attended daycare, of whom only a minority (27%) had received at least 1-dose of PCV7, the carriage rates for *S. pneumoniae* were 37% and 20% for *S. aureus* - however samples were nasal and not NP [24]. In contrast, children of mean age 5.3 years who had all received at least 1 dose of PCV9, the overall carriage rate of *S. pneumoniae, S. aureus* and *H. influenzae* were 54%, 35% and 57% respectively although carriage rates were significantly higher in HIV-infected children - 72% for *S. pneumoniae* and 74% for *H. influenzae* [25].These data are summarised in Table 3.

Table 3. PCV vaccination and subsequent carriage rate

Bacteria specific prevalence	Number of doses and study participants' age characteristics		
	PCV7 dosing trial, 2-30 months old	PCV7 - at least 1 dose 6 weeks – 4 years old	PCV9 -at least 1-dose mean age 5,3 years old
S. pneumoniae	70%	37%	54% (72% among HIV-infected infants)
S. aureus	-	20%	35%
H. influenzae	-	-	57% (74% among HIV-infected infants)
M. catarrhalis	-	-	-

Organism interactions

In Gambia among children <12 months old, positive interactions were observed between colonisation by *S. pneumoniae* with *H. influenzae* and *M. catarrhalis*; while a negative interaction between the colonisation by *S. pneumoniae* and *S. aureus* were observed [6]. Furthermore, it was noticed that there was an early colonisation by *H. influenzae* and *M. catarrhalis* which also frequently were co-carried alongside S. *pneumoniae*. In Sweden among children 1 – 6 years old, who experienced a penicillin-resistant *S. pneumoniae* there was a positive interaction between the colonisation of *S. pneumoniae* and *M. catarrhalis*

which decreased with age [11], while in this and a USA cohort, a negative interaction was observed between S. *pneumoniae* and *H. influenzae,* which became positive when *H. influenzae* co-colonised with *M. catarrhalis* [11, 1].

Meanwhile in Peruvian Andes children a positive interaction between colonisation by *S. pneumoniae* and *H. influenzae* and a negative interaction between colonisation by *S. pneumoniae* and *S. aureus* was seen [26]. A study distinguished between two levels of interactions [27], namely host (between subjects associations) and microbe (within subjects associations) and generally at both levels positive pairwise interactions were seen between *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*.

Furthermore, among healthy Indonesian children of ages 12 – 24 months old, findings showed positive interactions between *S. pneumoniae* and *H. influenzae,* and between *H. influenzae* and *M. catarrhalis*; a negative interaction between *S. aureus* and *M. catarrhalis* was recorded [17]. In younger children, aged 6 – 12 months, positive interactions were observed between S. *pneumoniae* with *H. influenzae* and *M. catarrhalis,* and a negative interaction was observed between colonisation by S. *pneumoniae* and *S. aureus* [28].

Additionally among healthy 6 -24 months old children, a positive interaction between colonisation of S. *pneumoniae* and *M. catarrhalis*, and a negative interaction between colonisation by S. *pneumoniae* and *S. aureus* was recorded, while those study participants who acquired AOM demonstrated negative interactions between colonisation by S. *pneumoniae* and *H. influenzae* and between colonisation by *H. influenzae* and *M. catarrhalis* [15]. Preschool children 2 – 5 years old, who had received antibiotics before undergoing an adenoidectomy for upper respiratory tract infection (URTI), were found to have bacteria in the NP which were not associated with bacteria from the adenoidectomy surgical process, although in the adenoids there was a positive interaction between colonisation by *S. pneumoniae* and *H. influenzae* [16].

Furthermore, in children aged 6 weeks - 4 years, colonisation by *S. pneumoniae* (vaccine-type and non-vaccine-type) were negatively associated with colonisation by *S. aureus* [24]. Children (mean age 5.3 years)who had received at least 1 dose of PCV9 and who were also HIV-infected, showed that *S. aureus* interacted negatively with *S. pneumoniae* and *H. influenzae* [25]. *Streptococcus pneumoniae* interacted positively with colonisation of *H. influenzae* among both HIV-infected and HIV-uninfected children [25].

It has been suggested previously through a review of clinical studies that the community of bacteria in the upper respiratory tract is altered by respiratory viruses, and that respiratory viruses may promote bacterial colonisation of the lower respiratory tract [29]. It is generally understood that viral infections precede or predispose one to subsequent bacterial superinfections (or later co-infections) [30], given this, Murphy et al. (2009) reviewed studies of interactions between upper respiratory tract viruses and NP pathogens in relation to pathogenesis of upper respiratory tract infections (URTIs). These researchers observed synergism between respiratory viruses and NP bacteria. A study of Australian Aboriginal and non-Aboriginal children [27] looked at interactions between respiratory bacteria and rhinovirus and adenovirus both of which both are associated with otitis media (OM). At pathogen/microbe level, rhinovirus showed association with *S. pneumoniae, H influenzae and M catarrhalis* while adenovirus showed association only with *M. catarrhalis* colonisation [27].

Meanwhile among children of ages 6 – 12 months old, NP colonisation of *S. pneumoniae* was positively associated with human rhinovirus and enterovirus; colonisation by *H. influenzae* was positively associated with human rhinovirus and respiratory syncytial virus; colonisation by *M. catarrhalis* was positively associated with coronavirus and adenovirus; and colonisation by *S. aureus* was positively associated with influenza viruses [28]. In South African children with AOM aged 3 months - 5 years old, about 77% - 79% of children in the study had NP colonisation by at least one virus, accompanied by a co-carriage with either *S. pneumoniae, H. influenzae* or *M. catarrhalis* sampled from middle ear fluid. Additionally, colonisation by *S. aureus* was detected alongside at least one virus from the NP in 63% of the study participants [15].

Previously applied statistical methods

As the summary of relevant literature indicates, a majority of the studies investigated NP carriage or colonisation from cross-sectional data. Cross-sectional studies are typically limited in the statistical methods that can be applied to analyse longitudinal data, and are unable to ascertain the temporality of carriage and co-carriage events.

A review of the literature revealed that the most common method of analysis was logistic regression which was used to estimate pairwise bacterial interactions with outcome variable as binary bacterial colonisation status [17]. Additionally, interaction estimates between pairs of species were analysed by Chi-square or Fisher's exact odds ratios (ORs) computed

whenever statistically appropriate [31] with one bacterial species colonisation as the outcome variable and the other species as the exposure variable.

Interpretation of such an interaction is the same as the ordinary interpretation of the OR, with OR > 1 taken to be an indication of a positive interaction and OR < 1 to be an indication of a negative interaction [31, 32]. However, Suzuki et al. (date) highlighted concerns about an approach using ORs to indicate organism interactions, and they advise that potential differences in risks of enrolment for colonisation-positive and colonisation-negative individuals should be incorporated into the computation of such ORs, especially for case-control studies. They argued that unless a population based cross-sectional study is used, one could anticipate a different enrolment risk for sick or healthy children. A further criticism of this approach is that unadjusted ORs are insufficient since each pairwise comparison may depend on interactions with yet other organisms [32].

Longitudinal designs allow for other approaches. The most common approach to exploring bacterial interaction in longitudinal studies were population averaged or marginal models known as generalised estimating equations (GEE). GEEs have been used for fitting both univariate and multivariate logistic regression models with bacterial carriage coded as a binary outcome (presence/absence) [1, 21, 31]. The main difference in these studies were the choices of correlation structure and that of covariates for adjustment in the multivariable models. While GEE models allow for efficient adjustment for correlated data, they do lead to population averaged estimates of association which may not be desired in all contexts.

A few studies fit conditional multivariable mixed effects models [27] to estimate associations between pairs of bacterial species. These models fit two random components, distinguishing between microbe level correlations and host level correlations, where microbe level represents within-subject correlations and host level represents between-subjects correlations. It has been argued that such a model has an advantage over analysing longitudinal data cross-sectionally because it accounts for correlations in repeated observations from the same study participant and is also capable of differentiating between within-level and between-level correlations.

Another method identified in longitudinal studies that was used to understand bacterial colonisation in terms of acquisition, clearance and change of state hazard rates, was the Markov transition model [33]. Lipsitch et al.(2012) used such a model to understand transitions of multiple *S. pneumoniae* serotypes with acquisition defined as any transition

from state 0 (not colonised with *S. pneumoniae*) to state *i* (any of the other *S. pneumoniae* serotypes), and with transition specific hazard rate and clearance defined as any transition from state *i* (resident state) to state 0. Similarly change of serotype could be specified as any transition from state *i* to state *j* (challenge state). With such a model approximating n^2+n independent rates and to reduce dimensionality, the authors [33] decided to fit four separate models each estimating 2n acquisition and clearance rates but limiting the change of states. Limitations of the model were acknowledged as assumptions that transition hazards are constant for each of the transitions, and that homogeneity of hosts may not reflect reality, considering for example that age is known to be a strong influence for clearance. Their model also did not adjust for simultaneous carriage of multiple serotypes.

Finally, it is worth drawing attention to the possibility of measurement error which may depend on the method used to identify the organisms. Differences in yield of bacteria arising from detection by culture or by polymerase chain reaction (PCR) are expected, and a number of studies have contrasted the disparity in yield of bacteria from these methods. For instance, in a study of children in their first year of life, the carriage rates of *S. pneumoniae, S. aureus, H. influenzae* and *M. catarrhalis* were all much higher in PCR samples compared to biological culture samples [26, 34].

Discussion

Interactions between disease-causing organisms is important from a public health point of view. Although the seven studies I reviewed are not really enough for accurate country to country comparisons, there were indications that *S. pneumoniae* carriage or colonisation is typically high in all geographic areas varying between 30-78%. The carriage pattern for *S. pneumoniae* suggests its prevalence might be high in infants after which it declines to moderate colonisation when the child is 24 months (median age). *S. aureus* carriage patterns suggest that carriage is at least moderate in younger children i.e. 50% in 2 months old infants thereafter decreasing with age. The carriages patterns of *H. influenzae* and *M. catarrhalis* suggest moderately low carriage at younger age namely 12% for *H. influenzae* in children younger than 24 months of age (in USA) and 32% for *M. catarrhalis* among 2 months old infants (in Gambia) rising to approximately 70% in children <12 months old.

Data, as shown in Table 2 suggest that colonisation by *H. influenzae* is independent of respiratory infection although it is very low among HIV-exposed infants. Colonisation rates

of *S. pneumoniae* and *M. catarrhalis* are relatively higher in children with other concomitant health conditions such as upper respiratory infections and acute otitis (AOM) and in HIV-exposed infants, compared to those with lower respiratory tract infections and AOM (recovered from middle ear fluid samples rather than NP).

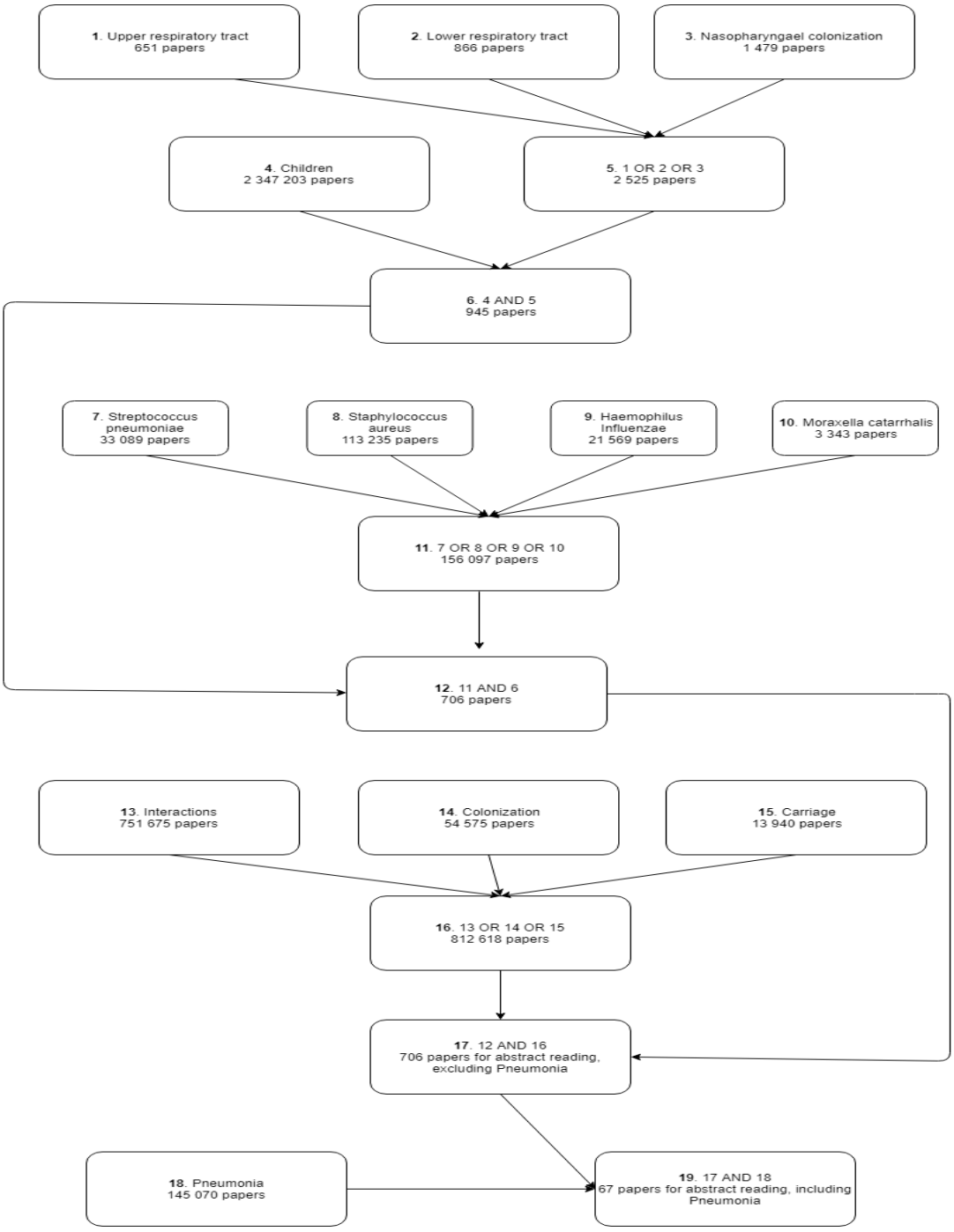
In Table 3, these data are not sufficient to support or refute the hypothesis of *S. aureus* increase post PCV vaccination by number of doses, however the PCV9 vaccine seem to be less effective among HIV-infected infants.

Positive interactions have been reported between *S. pneumoniae* and *M. catarrhalis,* while both positive and negative interactions have been reported with *H. influenzae* and *S. pneumoniae*. Positive interactions were recorded whenever *H. influenzae* and *M. catarrhalis* co-occurred. Among children with AOM, negative interactions have been described between *S. pneumoniae* colonisation wit*h H. influenzae* and between *H. influenzae* colonisation with *M. catarrhalis.* Meanwhile, negative interactions between colonisation by *S. aureus* with *S. pneumoniae* and *M. catarrhalis* have also been described. Moreover, among HIV-infected children *S. aureus* was also negatively associated with both *S. pneumoniae* and *H. influenzae,* while among HIV-infected and HIV-uninfected children *S. pneumoniae* and *H. influenzae* were both positively associated.

Cross-sectional methods used in longitudinal studies, for instance binning time into intervals, for calculations of odds ratios or interaction should be complemented by methods capable of controlling for a third variable for example other organisms interactions. Markov transition models seem ideal for investigating acquisition and clearance states and allow for the estimations for probability of change of states.

Appendix

Supplement Figure 1. Literature search strategy consort diagram



Literature Search Strategy.
Search terms: "Upper respiratory tract colonization", "Lower respiratory tract colonization", "Nasopharyngeal colonization", "Children", "Streptococcus pneumoniae", "Staphylococcus aureus", "Haemophilus influenzae", "Moraxella catarrhalis", "Interactions", "Colonization", "Carriage", "Pneumonia"

Supplement Table 1: Detailed search results

	Search term	**Date**	**Database**	**Results** (number of papers)
1	Upper respiratory tract	December 2018	PubMed	651
2	Lower respiratory tract	December 2018	PubMed	866
3	Nasopharyngeal colonisation	December 2018	PubMed	1 479
4	Children	December 2018	PubMed	2 347 203
5	1 or 2 or 3	December 2018	PubMed	2 525
6	4 and 5	December 2018	PubMed	945
7	Streptococcus pneumoniae	December 2018	PubMed	33 089
8	Staphylococcus aureus	December 2018	PubMed	113 235
9	Haemophilus influenzae	December 2018	PubMed	21 569
10	Moraxella catarrhalis	December 2018	PubMed	3 343
11	7 or 8 or 9 or 10	December 2018	PubMed	156 097
12	6 and 11	December 2018	PubMed	706
13	Interactions	December 2018	PubMed	751 675
14	Colonisation	December 2018	PubMed	54 575
15	Carriage	December 2018	PubMed	13 940
16	13 or 14 or 15	December 2018	PubMed	812 618
17	12 and 16	December 2018	PubMed	706
18	Pneumonia	December 2018	PubMed	145 070
19	17 and 18	December 2018	PubMed	67

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Part C: Manuscript

Title: Temporal interactions of microbiota in longitudinal nasopharyngeal samples

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Abstract

Background In the aetiology of bacterial pneumonia it is generally accepted that infection is preceded by nasopharyngeal (NP) colonisation. Several studies have shown that NP flora develop and establish in first year of life or early in childhood. Temporal organism interactions have rarely been explored due to the need for longitudinal data and the complexity of the statistical models for recurrent events.

Methods We sought to identify, describe, and quantify the temporal interactions existing between selected key bacteria colonising the nasopharynx in infants (up to 1 year of life). We then compared patterns in those children who go on to develop pneumonia versus those who do not. We applied multi-state models to examine the patterns of transition among states of colonisation while accounting for key confounders.

Results There were 760 individuals included in the analysis, with a total of 16,346 NP samples available and a median 364 person-days (IQR 346 – 365 person-days). There were temporally sustained positive interactions between *Streptococcus pneumoniae* with *Haemophilus influenzae; Streptococcus pneumoniae* with *Moraxella catarrhalis;* and *Haemophilus influenzae* with *Moraxella catarrhalis*. Moreover, the extent of association generally decreased with child age. *Staphylococcus aureus* had consistent negative interactions with other organisms.

Conclusions We have developed a flexible framework that characterised interactions between organisms while accounting for key time-varying confounders. We have demonstrated that multi-state models provide a useful approach to supplement classical modelling approaches.

Introduction

In the aetiology of bacterial pneumonia it is generally accepted that infection is preceded by nasopharyngeal (NP) colonisation [1]. Several studies have shown that NP bacterial flora develop and establish in the first year of life or early in childhood. Of the pathogens that colonise the nasopharynx the most commonly studied are *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis* and *Staphylococcus aureus*, in part because they are known contributors to lower respiratory tract infections [2].

Colonisation of the nasopharynx of young children is often asymptomatic [3], and concurrent with complex organism interactions. Colonisation is impacted upon by different risk factors such as antimicrobial treatment and/or vaccination [2], as well as environmental exposures, which may act as mediators.

A body of literature exists on organism interactions [1, 3-7].Temporal organism interactions have however not been explored in depth, especially regarding whether observed colonisation patterns are resultant of the host's immunity, synergism or antagonism between bacterial species interactions. An understanding of underlying temporal organism interactions in the nasopharynx may help predict how novel vaccines against these bacterial species may influence the ecology in this niche area.

We sought to identify, describe, and quantify temporal interactions existing between selected key bacteria present in the nasopharynx in infants (up to 1 year of life), and to compare these patterns in children who go on to develop pneumonia versus those who do not. We also explored methods for estimation of temporal interactions. We investigated patterns of carriage and co-carriage in a cohort of South African children residing in a peri-urban area near Cape Town, South Africa. We describe multivariable marginal models and multi-state models appropriate for the analysis of longitudinal data.

Participants and Methods

Study design

Data from the Drakenstein Child Health Study (DCHS) [15, 16] are used. DCHS is a population-based birth cohort study investigating early-life determinants of child health in a peri-urban setting in the Western Cape province of South Africa. The cohort includes more than 1000 mother-infant pairs engaged with the public sector health services from antenatal care until at least five years of age. Follow-up is ongoing- all infants have completed at least two years of follow up.

For this study, data come from nasopharyngeal (NP) swab samples taken every two weeks for the first year of life in a subset of more than 800 infants whose mothers enrolled in monitored particular cohort of the DCHS. In addition to routine mother-infant visits, study personnel actively surveyed episodes of illness, including lower respiratory tract infections (LRTI).

Data collection method

NP swabs were taken every two weeks and at LRTI episodes. Collected NP swabs (Copan flocked swab, FLOQSwabsTM, COPAN Diagnostics, Murrieta, CA, United States) were immediately placed into 1ml skim milk-tryptone-glucose-glycerol (STGG), transported at 4 °C to the laboratory within 2 hours of collection and frozen at −80 °C for later batch culture. Swabs were inoculated onto Mannitol Salt Agar (MSA) at the National Health Laboratory Services, Green Point Media Laboratory Cape Town, South Africa and incubated at 35 °C for 18–24 hours prior to measurement. The presence or absence of four organisms: *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis* and *Staphylococcus aureus* was recorded. Demographic and other data were collected by trained study personnel using electronic case report forms.

Data measures

Clinical and demographic parameters included sex of the child, date of birth, HIV status of mother and infant, and residential location. If an episode of lower respiratory tract infection occurred in the first year of life these data were recorded. Age groupings were used to stratify results for descriptive purposes and these included: birth-4 weeks, 5-12 weeks, 13-24 weeks and 25-52 weeks. These age categories were selected as they represent common stages in infant grown. All intervals are inclusive.

Carriage was defined on a per-sample and per-individual basis. Individuals were defined with positive carriage if they had any positive sample in the follow-up period. The frequency (percentage) of positive samples/individuals was calculated taking as the denominator the total samples available overall or in the specified time period. Co-carriage required the same sample to be a positive sample for both specified organisms.

Statistical analysis

Total person-days were calculated as the age (in days) at the last available sample for each individual. Age (in days) at the first positive sample was used to determine the age of first carriage. The cumulative time spent positive for carriage/colonisation was calculated as the sum of the intervals between consecutive positive samples (for each organism) including the following interval up to the first negative sample. Time regarded as negative was taken to be the individual total person-days minus the cumulative time regarded as positive.

Temporal pairs are defined as a pair of consecutive samples from the same individual. Clearance events are defined in a temporal pair, where the first sample is +ve (carriage) for a given organism and the second sample is –ve (no carriage). Acquisition events are the reverse (-ve → +ve). Steady state events are those remaining in a positive carriage state (+ve → +ve) or those remaining in a non-carriage state (-ve → -ve) over the temporal pair.

Generalised mixed effects models using a logit link were used to estimate the time varying association of organism carriage with the development of pneumonia, while enabling adjustment by key confounders. Crude and age-adjusted odds ratio (OR) and 95% confidence intervals were estimated for the positive-positive temporal interaction versus no association or positive-negative interaction.

Kaplan-Meier estimates were used to reflect time to first carriage. Markov multi state models (MSMs) were used to describe how organism acquisition in infancy rolls out with age and associated probabilities of clearance, new acquisition or persistent carriage. We fitted piecewise constant MSMs with knots fit at 28days (d), 84d, 168d and 365d corresponding to the *a priori* defined age categories. All MSMs were adjusted for age at sample collection and the allowable state-transitions are described in Supplement Figure 1. Observed versus expected prevalence plots were used to graphically assess model fit. Estimated transition probabilities were summarised numerically and graphically.

Ethical approval

All study procedures were carried out under approval by the University of Cape Town Human Research Ethics Committee for the DCHS study (HREC: 401/2009) and for this analysis (HREC: 240/2018). Participants in the original study provided written consent at recruitment and consent was updated annually if maternal participants wished to remain in the study.

Results

There were 760 individuals included in the analysis, with a total of 16,346 NP samples available and a median 364 person-days (IQR 346 – 365 person-days). *S. pneumoniae* was predominant, found in >55% of all samples and demonstrating carriage in >95% of individuals at least once by 12 months of age (Figures 1 and 2, Supplementary Table 1). *H. influenzae* and *M. catarrhalis* had 35% and 41% positive carriage respectively in all samples, and appeared at least once in >90% of individuals. *S. aureus* was less common (25% of samples and 88% of individuals) and also had a strikingly different pattern of first acquisition compared to the other three organisms (Figures 1 and 2), demonstrating a rapid increase in carriage prevalence until approximately 4 weeks and subsequently declining. *S. aureus* carriage appeared earlier than other bacterial species (Figure 2; Table 1) with first carriage event occurring at a median 28 days of age (IQR: 15 – 58 days) as compared to *H. influenzae* where first carriage occurred at a median 97 days of age (IQR: 57 – 155 days). Individuals remained positive for *S. aureus* for the shortest cumulative time, accruing a median 121 days (IQR: 55 – 180 days) and median 36% of the total person-days while both *H. influenzae* and *S. pneumoniae* had positive carriage for >200 days on average (Table 1).

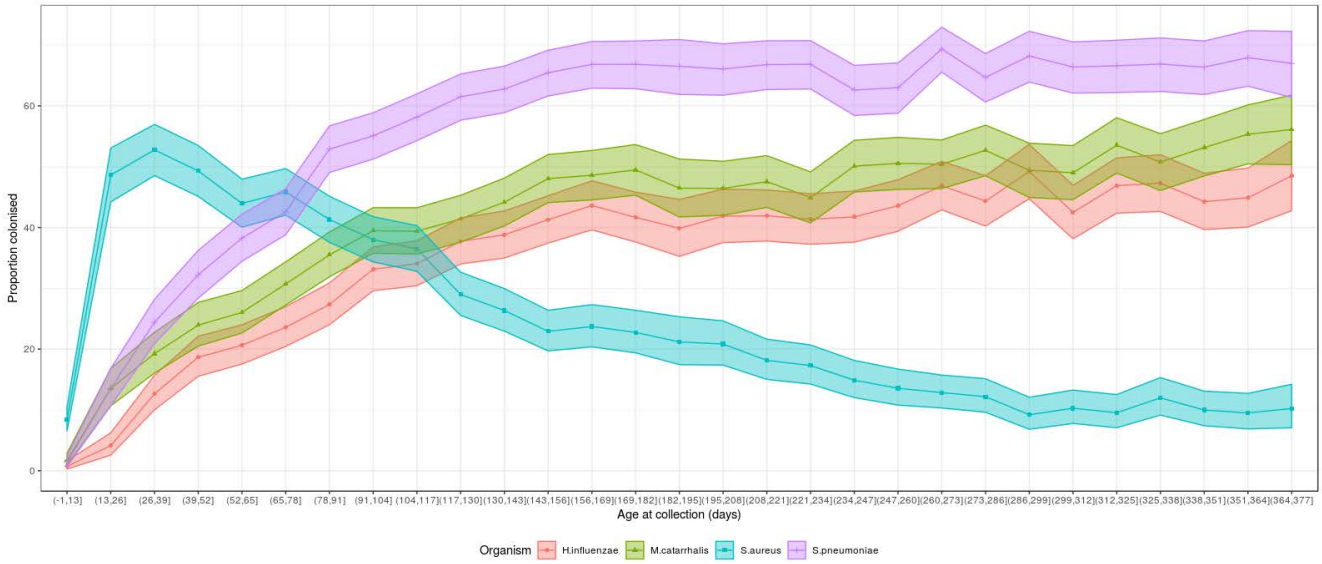

Figure 1. Prevalence (pointwise 95% CI) of sample carriage by each bacterial organism over time.

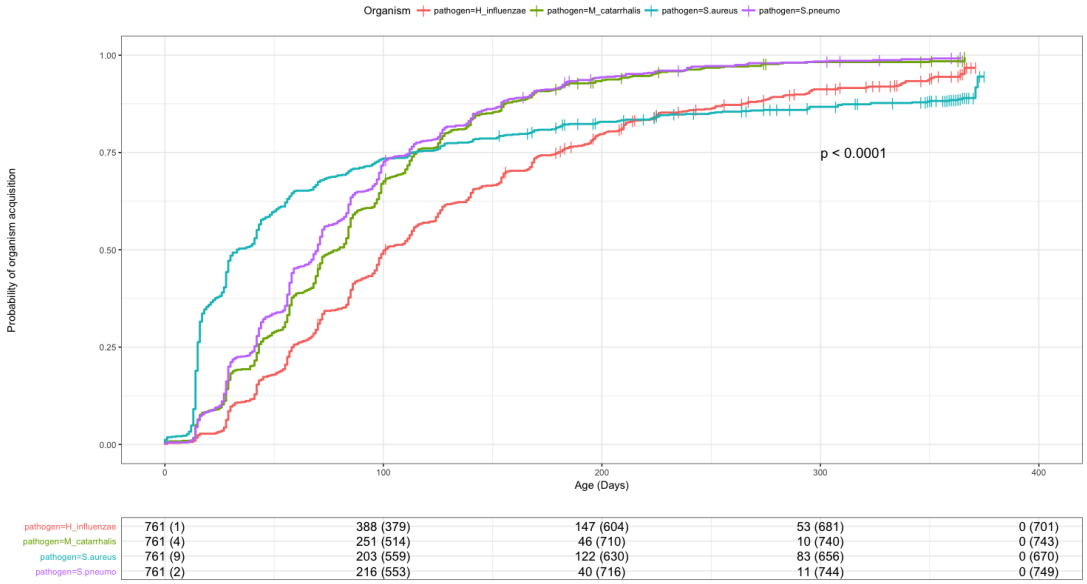

Figure 2. Cumulative probability of first carriage of bacterial organism by time (age in days).

Table 1. Statistical summary of estimated time spent with organism carriage (sum of person-days between consecutive positive samples for each individual) and the time spent without carriage (time spent positive subtracted from the total person-days for each individual)

Organism	Age (days) first carriage# Median (IQR)	Cumulative time (days) spent positive^ Median (IQR)	Percent person time positive Median (IQR)	Time (days) spent negative* Median (IQR)	Median (IQR) person-days
S. aureus	28 (15, 68)	121 (55, 180)	35.9% (16.0, 54.0)	219 (152, 286)	364 (346, 365)
S. pneumoniae	69 (41, 104)	246 (168, 301)	73.2% (52.9, 86.5)	88 (45, 156)	364 (346, 365)
H. influenzae	97 (57, 155)	168 (86, 238)	49.7% (26.7, 68.7)	169 (99, 239)	364 (346, 365)
M. catarrhalis	73 (43, 112)	200 (140, 264)	60.8% (43.9, 74.5)	127 (84, 193)	364 (346, 365)

among those ever positive; ^ Calculated as the median (IQR) of the individual sum of person-days between consecutive colonized samples including the right hand period (up to the first non-colonised sample). *Calculated as the median (IQR) of the individual person-days minus the individual days spent colonised

S. pneumoniae had the highest co-carriage prevalence with *H. influenzae* and *M. catarrhalis* (both 25%) but this varied by age category (Table 2). *H. influenzae* also co-occurred frequently with *M. catarrhalis* (19%). In contrast, *S. aureus* co-carriage was least prevalent with *S. pneumoniae* (12%), *H. influenzae* (5%) or *M. catarrhalis* (6%). Co-carriage frequencies differed considerably by age category, at least partially reflecting the relative prevalence of carriage by age. The co-carriage frequency of *H. influenzae* and *M. catarrhalis;* *H. influenzae* and *S. pneumoniae;* and *S. pneumoniae* and *M. catarrhalis*, increased with age,

from a rate of 4-6% in the first four weeks of life to 26-34% in the second 6 months of life. Co-carriage of *S. aureus* with all other organisms peaked between 5 and 12 weeks of age. Carriage and co-carriage rates were similar among those children who experienced LRTI compared to those who did not (Table 2). Seasonal carriage varied (Supplementary Figure 2), but to a lesser extent as compared to variance by age.

Overall, *M. catarrhalis* demonstrated the most stochastic or short term carriage over time, with 16% of temporal pairs representing clearance (carriage → absent) and 18% of temporal pairs being acquisition events (absent → carriage). This contrasts with *S. aureus* which had clearance events occurring in only 10% of temporal pairs and a similar rate for acquisition events (Supplementary Table 2).

S. aureus was negatively associated with all three bacterial species *S. pneumoniae* OR (95% CI): 0.68 (0.63 – 0.73), *H. influenzae* 0.41 (0.38 – 0.45) and *M. catarrhalis* 0.41 (0.38 – 0.45) in NP samples. Positive association interactions were evident between *S. pneumoniae* and *H. influenzae* OR (95% CI): 3.18 (2.97 – 3.41) and *S. pneumoniae* and *M. catarrhalis* 2.18 (2.04 – 2.32). *H. influenzae* and *M. catarrhalis* were also positively associated OR (95% CI): 2.58 (2.41 – 2.76).

Table 2. Percentage (count) of samples co-occurring by age category and LRTI status

Among all children							
Age category	**N**	**SA + SP**	**SA + HI**	**SA + MC**	**SP + HI**	**SP + MC**	**HI + MC**
Birth – 4 weeks	3019	10.2% (307)	3.88% (117)	5.3% (160)	5.17% (156)	6.36% (192)	4.21% (127)
5 – 12 weeks	2768	20.7% (572)	8.24% (228)	11.1% (307)	20.1% (557)	22% (609)	15% (416)
13 – 24 weeks	4416	14.4% (634)	6.5% (287)	6.75% (298)	30.8% (1361)	32.1% (1417)	23.2% (1024)
25-52 weeks	6143	6.85% (421)	3.48% (214)	4.49% (276)	33.7% (2068)	35.6% (2186)	26% (1598)
Birth – 1 year (overall)	16346	11.8% (1934)	5.18% (846)	6.37% (1041)	25.3% (4142)	26.9% (4404)	19.4% (3165)
Among children who experienced LRTI							
Age category	**N**	**SA + SP**	**SA + HI**	**SA + MC**	**SP + HI**	**SP + MC**	**HI + MC**
Birth – 4 weeks	1379	9.2% (127)	3.7% (51)	5.1% (70)	5.3% (73)	7.2% (100)	4.1% (56)
5 – 12 weeks	1307	20.6% (270)	7.5% (98)	10.9% (142)	17.8% (233)	21.3% (279)	15.1% (197)
13 – 24 weeks	2051	15.6% (319)	7.3% (149)	7.6% (155)	28.8% (590)	31.7% (650)	23.2% (476)
25-52 weeks	2838	7.3% (208)	3.3% (93)	4.3% (123)	32.7% (927)	36.2% (1026)	26.0% (738)
Birth – 1 year (overall)	7575	12.2% (924)	5.2% (391)	6.5% (490)	24.1% (1823)	27.1% (2055)	19.4% (1467)
Among children who did not experience LRTI							
Age category	**N**	**SA + SP**	**SA + HI**	**SA + MC**	**SP + HI**	**SP + MC**	**HI + MC**
Birth – 4 weeks	1640	11.0% (180)	4.0% (66)	5.5% (90)	5.1% (83)	5.6% (92)	4.3% (71)
5 – 12 weeks	1461	20.7% (302)	8.9% (130)	11.3% (165)	22.2% (324)	22.6% (330)	15.0% (219)
13 – 24 weeks	2365	13.3% (315)	5.8% (138)	6.0% (143)	32.6% (771)	32.4% (767)	23.2% (548)
25-52 weeks	3305	6.4% (213)	3.7% (121)	4.6% (153)	34.5% (1141)	35.1% (1160)	26.0% (860)
Birth – 1 year (overall)	8771	11.5% (1010)	5.2% (455)	6.3% (551)	26.4% (2319)	26.8% (2349)	19.4% (1698)

SA: *S aureus*; SP: *S pneumoniae;* HI: *H influenza*; MC: *M catarrhalis;* LRTI: lower respiratory tract infection

Mixed effects models adjusting for sex, site, season of birth, and age found temporally sustained positive associations between the co-carriages of *S. pneumoniae* with both *H. influenzae,* and *M. catarrhalis,* but no association with *S. aureus*. Moreover the extent generally decreased with infant's age (Table 3). Models including an indicator variable for LRTI in the first year of life had similar associations between organisms, and no evidence of meaningful associations with LRTI (Table 3).

Table 3. Associations with *S. pneumoniae* by generalised mixed effects models with a logit link and random effect term for individual. Each organism represents an independent model.

Models without LRTI status	**Main effects models^**		**Age interaction models^**	
	Est (SE)	**P-value**	**Est (SE)**	**P-value**
S. aureus	0.024 (0.05)	0.617	0.021 (0.05)	0.683
S. aureus * age interaction	-	-	-0.008 (0.05)	0.874
H. influenza	0.73 (0.04)	< 0.0001	0.86 (0.04)	< 0.0001
H. influenza * age interaction	-	-	-0.55 (005)	< 0.0001
M. catarrhalis	0.52 (0.04)	< 0.0001	0.54 (0.04)	< 0.0001
M. catarrhalis * age interaction	-	-	-0.51 (0.04)	< 0.0001
Models including LRTI status				
S. aureus	0.024 (0.05)	0.619	0.28 (0.07)	< 0.0001
S. aureus * age interaction	-	-	-0.009 (0.05)	0.864
LRTI in the first year of life	0.13 (0.10)	0.279	0.13 (0.12)	0.269
H. influenza	0.73 (0.04)	< 0.0001	0.79 (0.04)	< 0.0001
H. influenza * age interaction	-	-	-0.55 (0.04)	< 0.0001
LRTI in the first year of life	-0.01 (0.12)	0.934	-0.043 (0.12)	0.721
M. catarrhalis	0.52 (0.04)	< 0.0001	0.54 (0.04)	< 0.0001
M. catarrhalis * age interaction	-	-	-0.51 (0.04)	< 0.0001
LRTI in the first year of life	0.12 (0.12)	0.319	0.09 (0.12)	0.456

^ Adjusted for sex, site, season of birth, scaled age at collection, main effect for age retained in model and statistically significant for all models ($p < 0.0001$);

Multi-state models were built to estimate transition probabilities between co-carriage states, as well as estimate the association between predefined risk factors and transition between states. The general multi-state model scheme can be found in Supplement Figure 1. The model allowed for four possible states: no carriage, carriage with two organisms, or carriage with one organism and negative for the other. The multi-state process was assumed to be Markovian, with transition intensities modelled as piecewise constant.

The probability of acquisition of *S. pneumoniae* is modified by earlier carriage of *H. influenzae* or *M. catarrhalis*, however the reverse is not true. Positive *H. influenzae* carriage increases the probability of acquisition of *S. pneumoniae* with transition probabilities from 0.15 (95% CI 0.14-0.17) to 0.36 (95% CI 0.17, 0.54) for age categories over 28 days (Table 4), compared to the probability of acquisition of *S. pneumoniae* alone at 0.015 (95% CI 0.043-0.076) to 0.088 (95% CI 0.075-0.10) over the same age categories. This is in contrast to the probability of *H. influenzae* acquisition, which is similar with and without prior S. *pneumoniae* carriage. Similarly, carriage of *M. catarrhalis* increases the risk of *S. pneumoniae* acquisition (probability 0.24 vs 0.14 between 84 -168d of age), but *S. pneumoniae* carriage does little to modify the risk of *M. catarrhalis* acquisition. There was no evidence of differences between the acquisition of *S. pneumoniae* or *S. aureus* with relation to co-carriage.

There is no difference in the clearance of *S. pneumoniae* related to *H. influenzae* carriage, yet clearance of *H. influenzae* before 6 months of age is far less likely if coming from a state of co-carriage (probability between 0.04 - 0.07) compared to sole carriage (probability 0.23 - 0.12). The only evidence of differences in clearance probability in the models investigating *S. pneumoniae* and *M. catarrhalis* are in the probability of *M. catarrhalis* clearance before 28 days, which is 0.24 (95% CI 0.15 - 0.38) if carried alone and only 0.058 (55% CI 0.01 - 0.30) if carried with S. *pneumoniae,* though these confidence intervals overlap. Multi-state models

incorporating LRTI as a fifth absorbing state did not converge, nor did the secondary analysis of presented models on data subsets by LRTI status. Diagnostics for the presented models were accurate based on visual inspection of the predicted vs observed probability plots.

Table 4A. Predicted transition probabilities (95% confidence intervals) for the probability of state changes in each time period estimated with pairwise multistate models including S. *pneumoniae*, adjusted for sex, site, season of birth.

	Predicted probability (95% CI) of transition in the next 14 days with covariates at their means			
S. *pneumoniae with H. influenzae*	**0-28d**	**28-84d**	**84-168d**	**365d +**
Acquisition events				
Gain SP (from negative SP/HI)	0.019 (0.01, 0.034)	0.051 (0.043, 0.076)	0.066 (0.054, 0.080)	0.088 (0.075, 0.10)
Gain SP (from positive HI only)	0.062 (0.00, 0.13)	0.15 (0.14, 0.17)	0.21 (0.19, 0.22)	0.36 (0.17, 0.54)
Gain HI (from negative SP/HI)	0.067 (0.052, 0.086)	0.11 (0.098, 0.18)	0.13 (0.12, 0.15)	0.15 (0.13, 0.16)
Gain HI (from positive SP only)	0.011 (0.00, 0.35)	0.13 (0.095, 0.32)	0.19 (0.15, 0.22)	0.21 (0.19, 0.22)
Gain both (from negative SP/HI)	0.017 (0.00, 0.030)	0.013 (0.011, 0.78)	0.036 (0.029, 0.057)	0.050 (0.043, 0.069)
Clearance events				
Lose SP (from SP positive only)	0.37 (0.18, 0.63)	0.20 (0.00, 0.24)	0.19 (0.16, 0.23)	0.19 (0.17, 0.22)
Lose SP (from positive both)	0.35 (0.061, 0.66)	0.17 (0.13, 0.22)	0.15 (0.13, 0.17)	0.20 (0.18, 0.21)
Lose HI (from HI positive only)	0.23 (0.15, 0.38)	0.12 (0.00, 0.14)	0.087 (0.075, 0.10)	0.086 (0.076, 0.097)
Lose HI (from positive both)	0.044 (0.00, 0.57)	0.065 (0.043, 0.095)	0.063 (0.051, 0.078)	0.068 (0.060, 0.076)
Lose both	0.088 (0.048, 0.90)	0.051 (0.00, 0.089)	0.039 (0.030, 0.056)	0.043 (0.037, 0.053)
S. *pneumoniae with M. catarrhalis*	**0-28d**	**28-84d**	**84-168d**	**168+**
Acquisition events				
Gain SP (from negative SP/MC)	0.066 (0.051, 0.081)	0.088 (0.059, 0.10)	0.14 (0.12, 0.16)	0.16 (0.14, 0.18)
Gain SP (from positive MC only)	0.081 (0.00, 0.16)	0.18 (0.082, 0.28)	0.24 (0.15, 0.27)	0.31 (0.24, 0.33)
Gain MC (from negative SP/MC)	0.065 (0.052, 0.084)	0.10 (0.089, 0.29)	0.12 (0.11, 0.15)	0.12 (0.11, 0.15)
Gain MC (from positive SP only)	0.13 (0.01, 0.19)	0.10 (0.048,0.34)	0.14 (0.092, 0.17)	0.15 (0.12, 0.18)
Gain both (from negative SP/MC)	0.011 (0.00, 0.067)	0.020 (0.014, 0.69)	0.042 (0.033 ,0.096)	0.064 (0.050, 0.20)
Clearance events				
Lose SP (from SP positive only)	0.28 (0.17, 0.46)	0.26 (0.00, 0.33)	0.21 (0.18, 0.27)	0.19 (0.16, 0.23)
Lose SP (from positive both)	0.25 (0.074, 0.42)	0.26 (0.17, 0.32)	0.26 (0.20, 0.30)	0.29 (0.24, 0.31)
Lose MC (from MC positive only)	0.24 (0.15, 0.38)	0.099 (0.00, 0.20)	0.082 (0.070, 0.17)	0.090 (0.076, 0.17)
Lose MC (from positive both)	0.058 (0.01, 0.30)	0.068 (0.045, 0.097)	0.060 (0.049, 0.090)	0.063 (0.056, 0.090)
Lose both (from positive both)	0.060 (0.033, 0.86)	0.045 (0.00, 0.64)	0.030 (0.024, 0.45)	0.034 (0.030, 0.32)

Table 4B. Predicted transition probabilities (95% confidence intervals) for the probability of state changes in each time period estimated with pairwise multistate models including S. *pneumoniae* and *S aureus,* adjusted for sex, site, season of birth.

S. *pneumoniae with S aureus*	**0-28d**	**28-84d**	**84+ d**
Acquisition events			
Gain SP (from negative SP/SA)	0.22 (0.19, 0.25)	0.092 (0.077, 0.11)	0.059 (0.053, 0.067)
Gain SP (from positive SA only)	0.15 (0.00, 0.24)	0.12 (0.10, 0.16)	0.052 (0.00, 0.063)
Gain SA (from negative SP/SA)	0.057 (0.043, 0.074)	0.11 (0.091, 0.13)	0.19 (0.18, 0.21)
Gain SA (from positive SP only)	0.090 (0.00, 0.12)	0.11 (0.093, 0.14)	0.13 (0.00,0.16)
Gain both(from negative SP/SA)	0.020 (0.00, 0.073)	0.016 (0.013, 0.13)	0.013 (0.00, 0.17)
Clearance events			
Lose SP (from SP positive only)	0.097 (0.073, 0.21)	0.12 (0.099,0.14)	0.20 (0.17, 0.34)
Lose SP (from positive both)	0.063 (0.024, 0.19)	0.14 (0.11, 0.17)	0.29 (0.19, 0.31)
Lose SA (from SA positive only)	0.14 (0.070, 0.39)	0.11 (0.085,0.14)	0.097 (0.085, 0.15)
Lose SA (from positive both)	0.23 (0.15, 0.42)	0.081 (0.061,0.11)	0.077 (0.059, 0.090)
Lose both	0.028 (0.017, 0.74)	0.039 (0.024, 0.094)	0.031 (0.028, 0.74)

Discussion

Statistical modelling of temporal interactions may identify important organism level associations that change over time or vary in subgroups of children. We found positive and sustained interactions between *S. pneumonia* and both *H. influenzae* and *M. catarrhalis,* where our models indicated that preceding carriage or colonisation with either *H. influenzae* or *M. catarrhalis* may increase the risk of colonisation with *S. pneumonia.*

Timing of carriage and overall prevalence of carriage are in line with other findings in similar populations [1-7] with overall high exposure to *S. pneumonia*, *H. influenza* and *M. catarrhalis* during the first year of life and rapid and early exposure to *S. aureus.* Co-carriage is common, occurring in 5% - 26% of all samples, depending on the age of the children and the specific organisms. Similarly, clearance and acquisition events made up approximately 20% of all transitions suggesting that patterns of acquisition and clearance are dynamic. Carriage, co-carriage and transition frequency did not vary appreciably when comparing children who experienced LRTI in the first year of life versus those who had no lower tract respiratory infections. This suggests that the underlying pattern of exposure may be similar in

those children who do not experience LRTI in the first year of life, and so further investigation into the timing of exposure in the period before LRTI onset is critical.

The predicted transition probabilities from the multi-state models showed evidence of changing temporal risk of acquisition and clearance of *S. pneumonia* depending on co-carriage with other organisms.

This study has several strengths including an unprecedented sample frame of repeated NP samples in over 700 children during the first year of life and which accounted for children who experienced or did not experience lower respiratory tract infections (LRTIs). This represented a rare opportunity to carefully identify and analyse temporal interactions between organisms.

As an observational cohort, the positive associations that we found do not necessarily represent causal effects, and even though the temporal order was accounted for, organism carriage is not the same as colonisation or infection. Treatment with antibiotics and the impact of vaccination have not been accounted for here, and findings may change when considering colonisation with organisms rather than simple carriage, as well as adjustment for known environmental and clinical risk factors. The cohort is situated in a specific context and setting of South Africa, and so the results found here may not be generalizable to other settings.

We have presented an initial investigation into temporal interactions in a densely sampled longitudinal data set. Further analyses might consider different definitions of carriage, to get closer to the idea of colonisation, and account for washout periods due to antibiotics and/or other treatments. The multi-state models applied here have demonstrated their use as an important statistical tool in understanding longitudinal data and change between states over time.

Conclusion

Application of multi-state models to the temporal changes in nasopharyngeal carriage of four key disease-causing bacteria, has provided insights into the organism interactions occurring in the host. This work demonstrates that multi-state models are a useful additional statistical tool in cases where there are sufficient frequencies of longitudinal measures and repeat events.

Funding

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Manuscript supplement

Supplement Table 1: Percentage (frequency) of samples and individuals with organism carriage in age groups at the time of collection. Individuals were considered positive if they had any positive sample collected during the relevant age category.

Full cohort

		Samples					Individuals			
Age category	N samples	*SA*	*SP*	*HI*	*MC*	N	*SA*	*SP*	*HI*	*MC*
Birth – 4 weeks	3019	37.8% (1140)	20.4% (617)	10.7% (323)	15.7% (475)	730	65.9% (481)	45.9% (335)	26.3% (192)	39.9% (291)
5 – 12 weeks	2768	40.5% (1121)	51.9% (1436)	29.3% (811)	35.9% (994)	732	63.9% (468)	73.4% (537)	52.5% (384)	70.2% (514)
13 – 24 weeks	4416	23.7% (1048)	65.2% (2881)	40.9% (1805)	46.4% (2048)	734	56.0% (411)	91.1% (669)	76.3% (560)	87.7% (644)
25-52 weeks	6143	12.0% (737)	66.3% (4070)	44.8% (2752)	51% (3135)	684	49.4% (338)	94.0% (643)	82.5% (564)	91.1% (623)
Birth – 1 year (total)	16346	24.8% (4046)	55.1% (9004)	34.8% (5691)	40.7% (6652)	738	88.0% (650)	98.6% (728)	89.2% (685)	98.5% (727)

Among children who experienced LRTI

		Samples					Individuals			
Age category	**N samples**	***SA***	***SP***	***HI***	***MC***	**N**	***SA***	***SP***	***HI***	***MC***
Birth – 4 weeks	1379	36.3% (501)	19.8% (273)	10.2% (141)	16.9% (233)	318	65.0% (215)	45.6% (151)	27.2% (90)	42.6% (141)
5 – 12 weeks	1307	39.7% (519)	50.0% (640)	27.8% (364)	35.6% (466)	337	61.1% (204)	71.6% (239)	52.1% (174)	70.4% (235)
13 – 24 weeks	2051	25.7% (527)	63.0% (1293)	40.7% (835)	47.9% (982)	334	59.1% (199)	91.4% (308)	76.6 % (258)	89.0% (300)
25-52 weeks	2838	11.7% (333)	65.9% (1871)	44.0% (1298)	51.6% (1466)	331	49.7% (158)	94.7% (301)	85.5% (272)	90.3% (287)
Birth – 1 year (total)	7575	24.8% (1880)	53.8% (4077)	34.1% (2588)	41.5% (3175)					

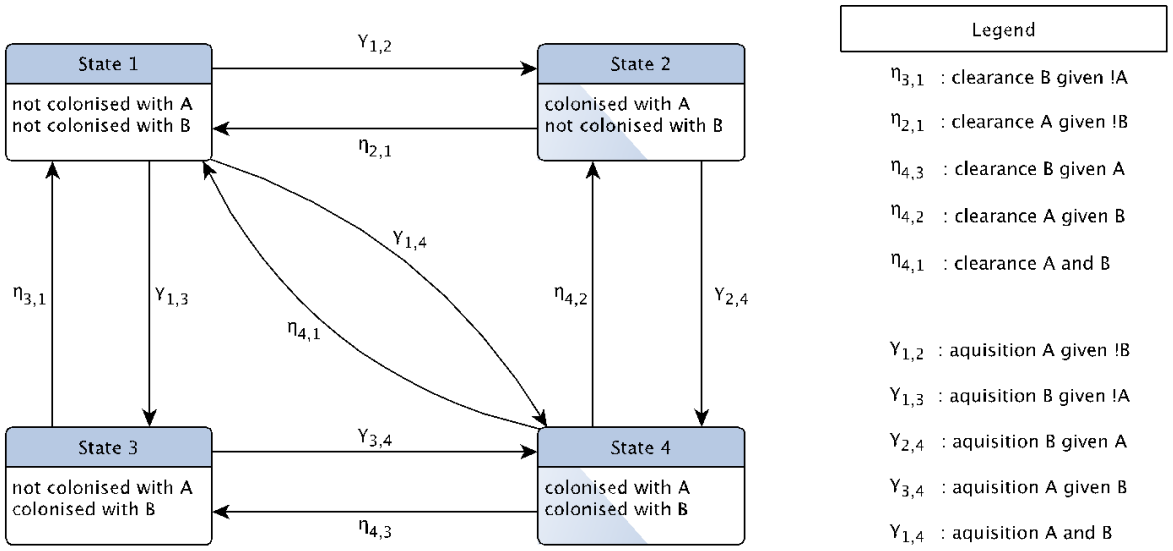
Among children who never experienced LRTI

		Samples					Individuals			
Age category	**N samples**	***SA***	***SP***	***HI***	***MC***	**N**	***SA***	***SP***	***HI***	***MC***
Birth – 4 weeks	1640	39.0% (639)	21.0% (344)	11.1% (182)	14.8% (242)	399	66.7% (266)	46.1% (184)	25.6% (102)	37.6% (150)
5 – 12 weeks	1461	41.2% (602)	54.5% (796)	30.6% (447)	36.1% (528)	398	66.3% (264)	74.9% (298)	52.8% (210)	70.1% (279)
13 – 24 weeks	2365	22.0% (521)	67.1% (1588)	41.1% (970)	45.1% (1066)	397	53.4% (212)	90.9% (361)	76.1% (302)	86.6% (344)
25-52 weeks	3305	12.2% (404)	66.5% (2199)	45.5% (1504)	50.5% (1669)	366	49.2% (180)	93.4% (342)	79.8% (292)	91.8% (336)
Birth – 1 year (total)	8771	24.7% (2166)	56.2% (4927)	35.4% (3103)	40.0% (3505)					

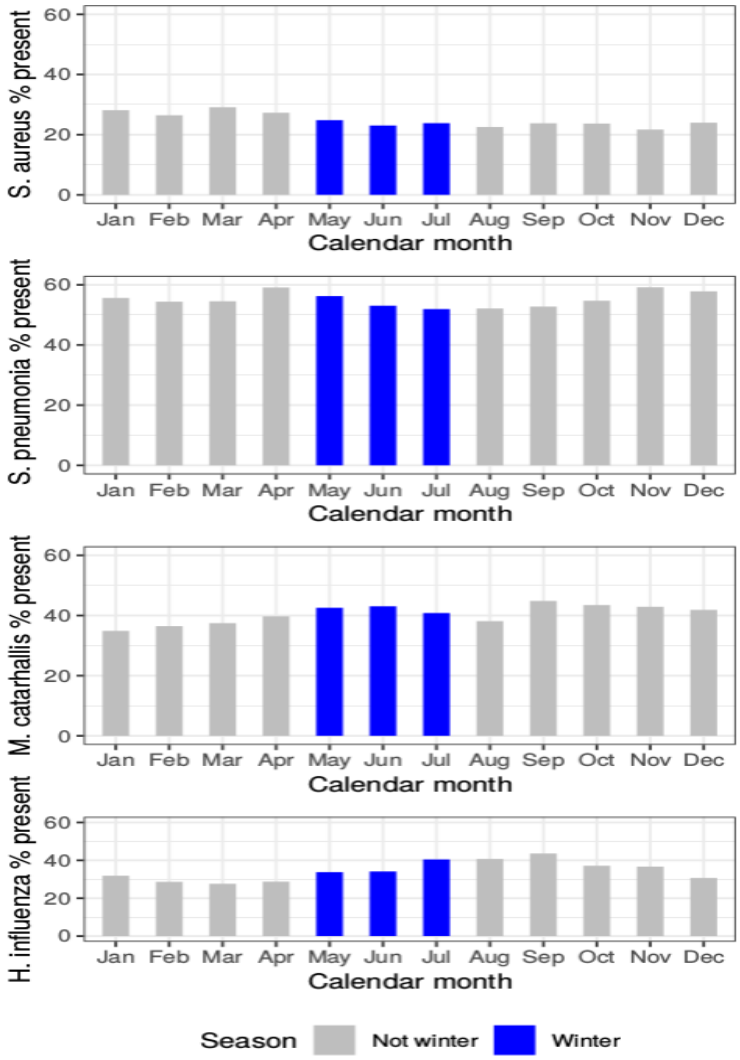
SA: *S aureus*; SP: *S pneumoniae;* HI: *H influenza*; MC: *M catarrhalis;* LRTI: lower respiratory tract infection

Supplement Table 2. Percentage (frequency) of each pair of sequential samples (n = 15,608 pairs) that had a clearance, acquisition or remained in existing state from observation *t* to observation *t+1*.

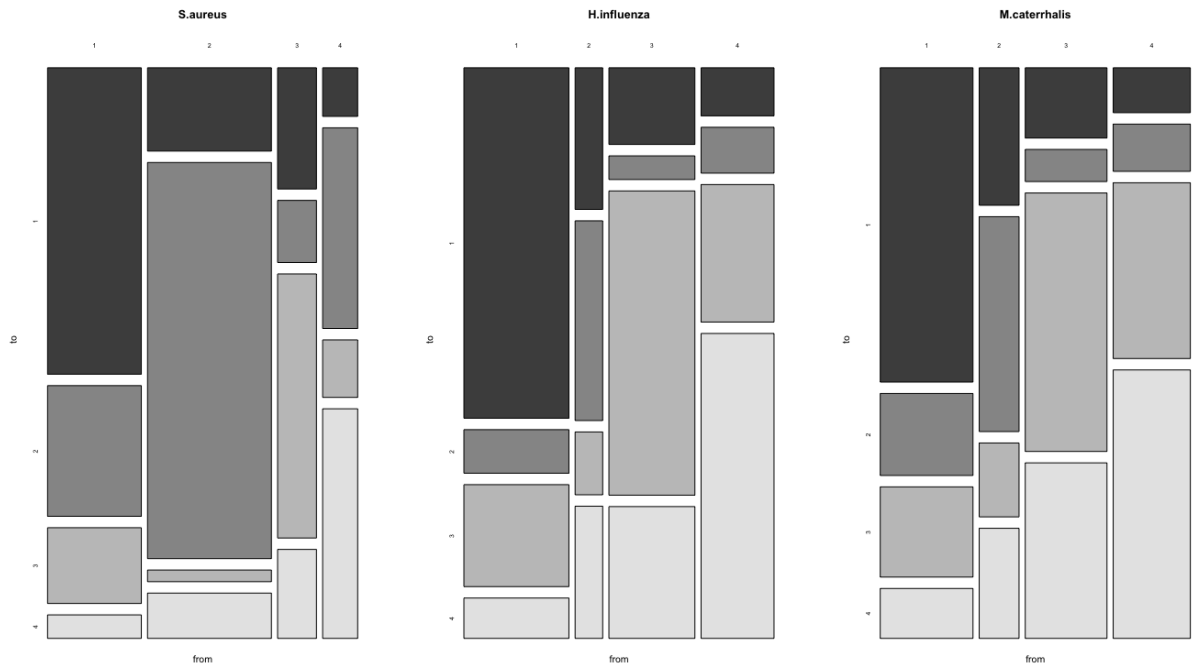
	S. aureus	***S. pneumoniae***	***H. influenzae***	***M. catarrhalis***
Clearance **+ve → -ve**	9.7% (1583)	9.5% (1545)	11.7% (3468)	15.6% (2541)
Acquisition **-ve → +ve**	10.0% (1633)	12.4% (2029)	13.6% (2214)	17.9% (2915)
No change **-ve → -ve**	61.5% (10019)	31.1% (5071)	49.2% (8012)	39.4% (6427)
No change **+ve → +ve**	14.6% (2373)	42.7% (6963)	21.3% (3468)	22.8% (3725)



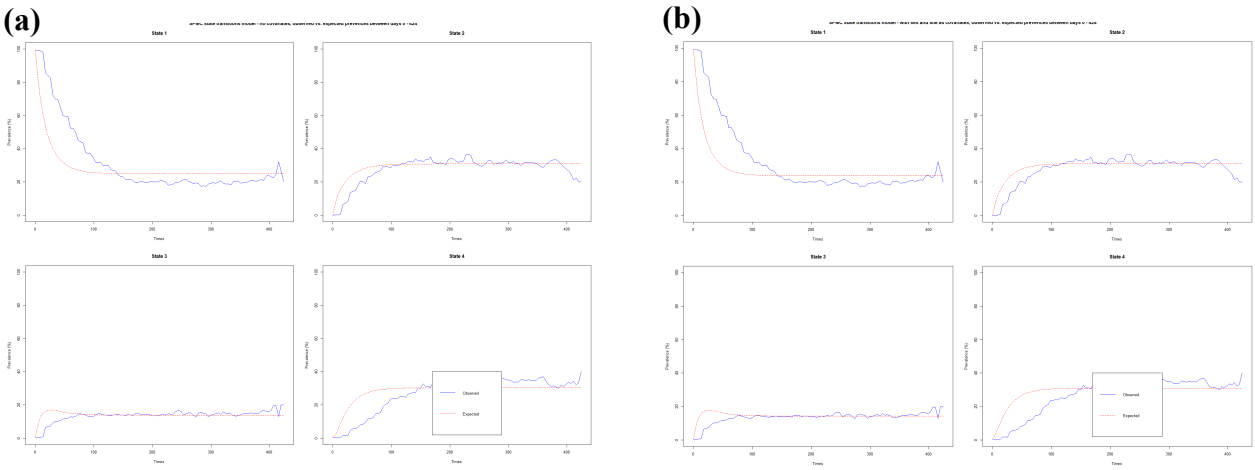
Supplement Figure 1. Schematic of two-organism states and transitions. Some organism pairs had additional transitions non-allowable due to small numbers.



Supplement Figure 2. Seasonal carriage by organism and calendar month.



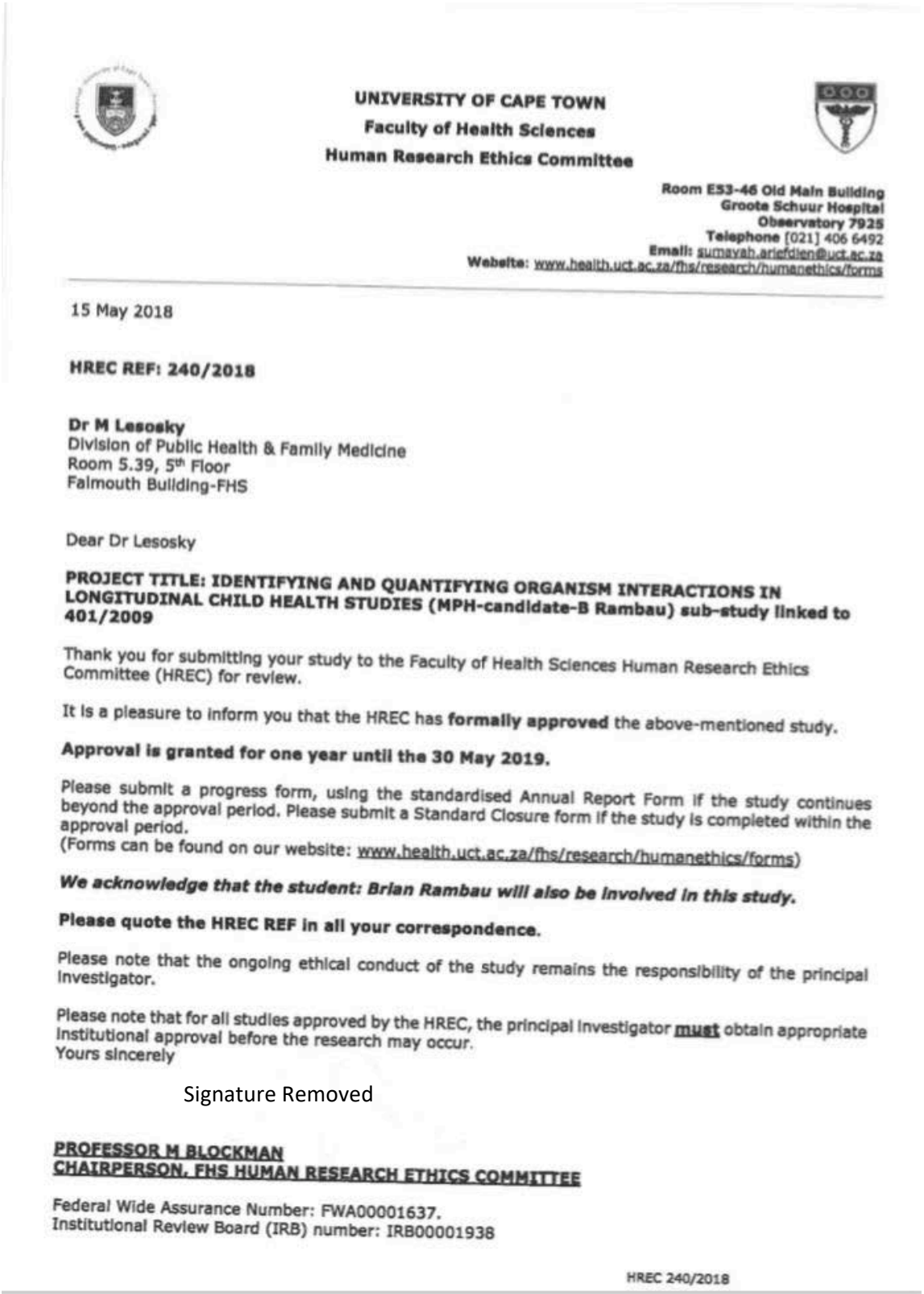
Supplement Figure 3. Mosaic plot of co-colonisation transition matrix for *S. pneumonia* with other organisms. Rows represent origin state (time *t*), columns represent next state (time *t+1*) and size reflects probability of transition.



Supplement Figure 4. Selected model diagnostics for multi-state models baseline (a) and covariate adjusted (b) for the *S. pneumonia* and *M. catarrhalis* model.

Part D: Appendices

Appendix 1: HREC approval letters



UNIVERSITY OF CAPE TOWN
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Website: www.health.uct.ac.za/fhs/research/humanethics/forms

15 May 2018

HREC REF: 240/2018

Dr M Lesosky
Division of Public Health & Family Medicine
Room 5.39, 5th Floor
Falmouth Building-FHS

Dear Dr Lesosky

PROJECT TITLE: IDENTIFYING AND QUANTIFYING ORGANISM INTERACTIONS IN LONGITUDINAL CHILD HEALTH STUDIES (MPH-candidate-B Rambau) sub-study linked to 401/2009

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee (HREC) for review.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30 May 2019.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

We acknowledge that the student: Brian Rambau will also be involved in this study.

Please quote the HREC REF in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval before the research may occur.
Yours sincerely

Signature Removed

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE

Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938

HREC 240/2018

UNIVERSITY OF CAPE TOWN

FACULTY OF HEALTH SCIENCES
Human Research Ethics Committee

FHS016: Annual Progress Report / Renewal

HREC office use only (FWA00001637; IRB00001938) This serves as notification of annual approval, including any documentation described below.			
☑ Approved	Annual progress report	Approved until/next renewal date	30/08/19
☐ Not approved	See attached comments		
Signature Chairperson of the HREC	Signature Removed pp	Date Signed	12/09/2018

Comments to PI from the HREC

Principal Investigator to complete the following:

1. Protocol Information

Date (when submitting this form)	22 Aug 2018		
HREC REF Number	401/2009	Current Ethics Approval was granted until	30 Aug 2018
Protocol title	Drakenstein Child Health Study		
Protocol number (if applicable)	Protocol v1.15		
Are there any sub-studies linked to this study?	✓ Yes ☐ No		
If yes, could you please provide the HREC Ref's for all sub-studies? **Note:** A separate FHS016 must be submitted for each sub-study.	Details included in appendix.		
Principal Investigator	Prof Heather Zar		
Department / Office Internal Mail Address	Department of Paediatric and Child Health Red Cross War Memorial Children's Hospital		

(Note: Please complete the Closure form (FHS010) if the study is completed within the approval period)

Appendix 2: Instructions for Authors –Annals of Epidemiology

ANNALS OF EPIDEMIOLOGY

TABLE OF CONTENTS

AUTHOR INFORMATION PACK

ISSN: 1047-2797

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INTRODUCTION

Annals of Epidemiology is a peer reviewed, international journal devoted to epidemiologic research and methodological development. The journal emphasizes the application of epidemiologic methods to issues that affect the distribution and determinants of human illness in diverse contexts. Its primary focus is on chronic and acute conditions of diverse etiologies and of major importance to clinical medicine, public health, and health care delivery. *Annals* encourages the use of epidemiology in a multidisciplinary approach to understanding disease etiology. The *Annals* publishes Original articles, Brief Communications, Rapid Reports, Reviews, Commentaries, Editorials, Letters to the Editor, and Book Reviews.

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Brief Communication: a short Original Article--<=2000 words; structured abstract <=200 words (Purpose, Methods, Results, Conclusions), <=3 Tables, Figures, or other illustrative materials. For Article Type at submission, please select Short Communication, then Brief Communication as the manuscript category.

Rapid Reports: a new fact, method, observation for quick presentation–<=1000 words; no abstract; maximum of one table, figure, or other illustrative materials. For Article Type at submission, please select Short Communication, then Rapid Reports as the

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